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Viability of *Bacillus subtilis* Spores in Rocket Propellants

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ABSTRACT

GODDING, ROGENE M. (Lockheed Missiles & Space Co., Palo Alto, Calif.), AND VICTORIA H. LYNCH. Viability of *Bacillus subtilis* spores in rocket propellants. Appl. Microbiol. **13**:10-14. 1965.—The sporicidal activity of components used in liquid and solid rocket propellants was tested by use of spores of *Bacillus subtilis* dried on powdered glass. Liquid propellant ingredients tested were N_2O_4, monomethylhydrazine and 1,1-dimethylhydrazine. N_2O_4 was immediately sporicidal; the hydrazines were effective within several days. Solid propellants consisted of ammonium perchlorate in combination with epoxy resin (EPON 828), tris-1-(2-methyl) aziridinyl phosphine oxide, bis-1-(2-methyl) aziridinyl phenylphosphine oxide, and three modified polybutadiene polymers. There was no indication of appreciable sporicidal activity of these components.

A major objective of space exploration on other planets is a search for evidence of extraterrestrial life. Sterilization of all components of the space vehicle and associated equipment is essential to avoid contamination of these planets with earth organisms that might invalidate these investigations. The International Committee on Contamination by Extraterrestrial Exploration (1958) has recommended that all efforts be made to avoid biological contamination of the moon and other celestial bodies. A study group, sponsored by the National Academy of Sciences and National Research Council (1962), has recommended that sterility be an absolute requirement for all space vehicles that approach or land on Mars. This requirement remains an official position of the National Aeronautics and Space Administration (Nicks and Reynolds, 1963).

Rocket propellants are one of the components of a spacecraft that must be sterile. When the propellant components are activated, sterilizing temperatures are achieved. However, until these materials are mixed and react chemically, they remain a potential source of interplanetary contamination if not sterile. This paper considers the problems of testing these materials for sterility, and presents data for the sterilizing properties of some rocket propellants against *Bacillus subtilis*.

MATERIALS AND METHODS

Chemicals. Components of liquid propellants were: monomethyl hydrazine (MMH), Matheson Co., Inc., East Rutherford, N.J. (technical grade); 1,1-dimethyl hydrazine (UDMH), Matheson Co., Inc., (technical grade); and nitrogen dioxide (N_2O_4), Matheson Co., Inc., (99.5% minimum).

Components of solid propellants were: ammonium perchlorate (NH_4ClO_4), American Potash & Chemical Corp., Los Angeles, Calif. (sizes: 48 mesh and 10 μ); tris-1-(2-methyl aziridinyl phosphine oxide (MAPO), Interchemical; bis-1-(2-methyl) aziridinyl phenylphosphine oxide (P-MAPO), Interchemical; EPON Resin 828, Shell Chemical Co., New York, N.Y.; polybutadiene-acrylonitrile-acrylic acid terpolymer (PBAN); polybutadiene-acrylic acid copolymer (PBAA); and carboxyl-terminated polybutadiene (CTPB).

Spore glass preparations. Spore suspensions of *B. subtilis* were prepared as follows. Potato-agar plates were inoculated with 0.1 ml of an 8-hr culture growing on Difco Antibiotic Medium #3 (penicillin assay broth). After incubation at 37 C for 2 to 5 days, sporulation had occurred. The cell mass was scraped off the agar and resuspended in 10 ml of sterile water. Vegetative cells were killed by placing the cell suspension in a water bath 80 C for 10 min, followed by rapid cooling. The resulting spore suspension was stored at 5 C. The titer of these preparations was between 10^8 and 10^{10} viable spores per milliliter.

A dry suspension of spores on powdered glass was prepared by use of Corning glass #7574 with a particle-size range of 5 to 150 μ in diameter. A sample of the liquid spore suspension was added to 10 g of heat-sterilized glass, to give approximately 10^6 spores per 10 mg of powdered glass. After thorough mixing, the slurry of spores and glass was dried in a vacuum desiccator over P_2O_5. The dried spore-glass preparation was stored at 5 C. Sterile equipment and aseptic procedures were used at each step of the preparation.

Sporicidal test procedure. A 10-mg amount of the dry spore-glass mixture was added to 1 g of the propellant component being tested. Exposures were performed at room temperature.

Liquid propellant components. The test exposures for MMH and UDMH were performed in 20-mm screw-cap test tubes. To give uniform exposure to all spore-glass particles, the tubes were constantly rotated on a revolving roller drum. At the end of the exposure time, the glass-spore-propellant mixture was withdrawn from the tubes with a Pasteur pipette and placed in a sterilized flask, which was immediately attached to a rotator connected to a duo-valve vacuum pump with a liquid-nitrogen trap. The flask was rotated in a water bath maintained at 40 C. Evaporation of the liquid required an average time of 2 min; in some cases, evaporation was complete in 30 sec, and occasionally it required as long as 10 min. After evaporation, 1 ml of sterile water was added to the flask, the flask was shaken vigorously to resuspend the glass and spores, and 0.1 ml of the resultant suspension was plated on a nutrient agar plate. Further dilutions were made, and 0.1 ml of each dilution was plated in duplicate on nutrient agar plates to establish a survival curve. The total operation of transfer of the test mixture from test tube to evaporation flask through final plating required approximately 10 min.

The glass-spore mixture to be exposed to N_2O_4 was placed at the bottom of sterile 20-mm screw-cap tubes with Teflon lining the entire cap. The N_2O_4 was introduced directly into the tubes from a cylinder containing N_2O_4 under pressure, and was either condensed in cold water (18 C) or allowed to remain as a gas phase. Collecting from the tank required approximately 30 sec. Treatment during the exposure time was identical to the method used for the hydrazines. After the exposure period, the N_2O_4 was removed by flushing the tube with a sterile stream of N_2 for 2 min. The spore-glass mixture was resuspended in 1 ml of water, and the entire sample was plated on nutrient agar.

Solid propellant components. Solid NH_4ClO_4, MAPO, or P-MAPO, and the glass-spore mixture were intimately mixed in sterile aluminum weighing pans. After the exposure period, 10 ml of water were added to the mixture and swirled until the propellant component was dissolved and the glass and spores were resuspended (approximately 1 min). Dilutions were made, and each dilution was filtered through a membrane filter. The filters were placed on nutrient agar and incubated at 37 C for 48 hr.

PBAN, PBAA, CTPB, and EPON are water-insoluble and very viscous materials. After stirring to mix the propellant components and spores, the spores were recovered from the viscous mixture by the following procedure. Water (10 ml) was added and blended at low speed for 2 min with a VirTis mixer with a macro shaft and blade (The VirTis Co., Inc., Gardiner, N.Y.). The mixing was done either in sterile aluminum pans or in plastic disposable beakers. The VirTis blade and shaft were flamed in alcohol to sterilize. Covers to fit over the mixing pan were made from plastic petri dish covers with holes drilled in the centers to fit the shaft. These were then sterilized in ethylene oxide. Appropriate dilutions of the aqueous phase were filtered through membrane filters, which were then placed on nutrient agar plates and incubated at 37 C for 48 hr. To detect late germination of spores, the filters were kept for an additional 7 to 10 days at room temperature. There were no changes in total colony number on any of the test filters.

Test for toxicity of residual solids. If colonies did not appear on the nutrient agar-filter plates after 48 hr of incubation, the plates were tested for the presence of toxic materials. A drop of dilute suspension of spores of *B. subtilis* was dropped on the filter and on the agar, and the plate was reincubated for 48 hr. The presence of toxic materials on the filter was indicated by growth on the agar and absence of growth on the filter.

RESULTS

Survival of spores on glass powder. Testing of the sporicidal properties of the materials required that the test simulate as closely as possible conditions that will prevail during real operation. Under actual field conditions, dry microorganisms would come into contact with dry compounds. Therefore, spores dried on glass were used as a means of adding a uniform number of spores to each test chemical without addition of water or other liquids. Table 1 presents the results of survival of these spores at 25 and 3 C. Survival of the spores at 25 and 3 C was similar. During a storage period of 66 days, the mean spore count was 2.3×10^5 with a standard deviation of 0.73×10^5. Therefore, during the test periods investigated, a reduction in the viable cell number outside the standard deviation of the control is an

TABLE 1. *Survival of spores on glass powder**

Storage temp	No. of viable spores $\times 10^5$ after storage for								
	0 days	1 day	2 days	3 days	6 days	10 days	13 days	43 days	66 days
C									
25	4 1.6	2 1.3	1.9 3.3	1.7	2.5	2	2.8	1.8	2.1
3	4 1.6	1.5 2.7	2.5 1.6	2	1.5	2.9	2.5	2.1	3.9

* Viable spores of *Bacillus subtilis* (8×10^7) added to 1 g of Corning glass #7574 and dried over P_2O_5. Samples (10 mg) stored in glassine packages. Values recorded are averages of duplicate plates. Growth on nutrient agar at 37 C in 48 hr.

effect of the test exposure and not a decline in the viability of the spore preparation.

Liquid propellants. A liquid propellant is formed by adding N_2O_4 to MMH or UDMH. These compounds are stored in separate compartments until used.

The sporicidal properties of nitrogen dioxide were tested by exposing 10 mg of the spore-glass preparation containing 6×10^5 spores of *B. subtilis* to the vapor of N_2O_4 at 25 C. The N_2O_4 was immediately removed with a stream of N_2. Exposure of spores to N_2O_4 was less than 5 min. In three experiments, no viable spores were recovered. Visual examination of the plates was made 48 hr after incubation at 37 C and periodically during 1 week of storage at room temperature.

The results of exposure of dried spores to the hydrazines are presented in Table 2. Both MMH and UDMH have sporicidal properties. MMH is a more effective sporicidal compound than is the UDMH. Killing is not instantaneous, but requires an exposure of several days for some spore preparations.

Testing of individual components of solid propellants. A representative type of solid propellant to be used on interplanetary travel was investigated. Three varieties of propellants were tested: (i) polycarbutene N made up of solid ammonium perchlorate (NH_4ClO_4), an epoxy resin (EPON 828), and PBAN terpolymer; (ii) polycarbutene R made up of NH_4ClO_4, MAPO, P-MAPO, and PBAA copolymer; and (iii) NH_4ClO_4, MAPO, P-MAPO, and CTPB polymer. The sporicidal properties of each component were tested (Table 3).

Exposure of dry spores to dry ammonium perchlorate produced little or no killing over a period of 120 hr. The average value of the recovered spores from the two mesh sizes of ammonium perchlorate immediately after mixing was only slightly lower than the initial titer of

TABLE 2. *Exposure of dried spores to hydrazine*

Hydrazine	Expt	Spore age	Spores per ml	Exposure time (hr)								
				0*	0.25	0.5	1	1.5	2	6	26	52
		months										
Monomethyl hydrazine	1	8	3×10^3					0				
	2	9	4.4×10^3						0			
	3	9	7.1×10^5	6.3×10^5†	7.4×10^5	10^5	10^3	4.6×10^2	3.5×10^2			
	4	1	1×10^6	0	0	0	0	0	0			
	5	1	1.6×10^5	8×10^4	0		0					
	6	1	1.6×10^5	0	0	0	0					
1,1-Dimethyl hydrazine	1	8	3×10^3						0			
	2	9	5.3×10^3						10^1			
	3	1	2×10^5	4.4×10^4		10^5	6×10^2	6.7×10^4	7.8×10^3	4.6×10^4		
	4	1	1.8×10^5	1.4×10^4			1.3×10^4	1.7×10^3	1.9×10^3	2×10^2	2×10^2	0

* Zero time plus the time required to evaporate test chemical from spores.

† Numbers indicate number of spores per milliliter after indicated exposures.

TABLE 3. *Survival of spores in solid propellant components**

Propellant component	Exposure time (hr)				
	0	2	72	96	120
NH_4ClO_4, 48 mesh	4.6×10^5	3×10^5	2.3×10^5	2.3×10^5	2.1×10^5
NH_4ClO_4, 10 μ	6.3×10^5	5×10^5	$\geq 10^5$	4×10^5	2.2×10^5
EPON	4×10^2	1.9×10^3	8.8×10^2	9.5×10^2	
MAPO	$\geq 10^5$	3.1×10^5		3.1×10^5	2.9×10^5
P-MAPO	$\geq 10^5$	2.7×10^5		2.1×10^5	1.6×10^5
CTPB	2.5×10^3	9.2×10^2		0.8×10^2	2.8×10^2
PBAN (1)	3.2×10^3	4×10^2	10^2	$<10^2$	
(2)	$<10^2$	8×10^2	1.6×10^4	$<10^2$	
PBAA (1)	1.7×10^3	7.4×10^3		2×10^2	$<10^2$
(2)	$<10^2$	$<10^2$		2.1×10^3	2.4×10^4

* Spore-glass mixture (10 mg) containing 6.2×10^5 *Bacillus subtilis* spores added to 1 g of component. The symbol < indicates that the filter from dilution 10^{-2} was not toxic to growth but showed no colonies, whereas dilutions 10^{-1} and 10^0 were toxic (see Toxicity test). Exposure temperature, −25 C. Results are expressed as number of viable spores.

the spore-glass mixture. Samples analyzed after 72, 96, and 120 hr showed a constant value slightly lower than the original titer. These experiments indicate that, in the dry state, ammonium perchlorate is not sporicidal to any appreciable extent.

EPON 828 is an epoxy resin of high viscosity at room temperature. The dry spore-glass mixture was intimately mixed with this resin. After the exposure period, recovery of viable spores was attempted by vigorous mixing with a high-speed VirTis blender. Visual examination of the suspension indicated incomplete mixing of the solid and liquid phase. Therefore, this extraction procedure gives low recovery of spores at zero time (see Table 3); 4×10^2 spores were recovered from an initial 6×10^5 spores. Succeeding samples showed extreme variability with higher values than the zero-time viability. After consideration of the sampling problems, we feel that the observed decrease from 10^5 to 10^2 is probably a reflection of inability to recovery spores from the viscous resin rather than the result of killing by the EPON resin.

MAPO and P-MAPO are substituted phosphine oxides. They are miscible with water at the concentrations used in these tests. Recovery of spores with these compounds was comparable to that obtained with ammonium perchlorate. No decrease in viable-spore recovery was observed with increased exposure time.

PBAN, PBAA, and CTPB are viscous liquid polymers used as binders in propellants (see chemical description). Mixing of spores with these materials was difficult, and recovery of spores for testing of viability was unsatisfactory. Fewer that 1% of the added spores were recovered at zero time after mixing with the modified polybutadiene polymers. During the test period of 120 hr, only a slight decrease in spore recovery was observed. These results demonstrate that the individual components of the solid propellants tested are not sporicidal.

Testing of mixture as prepared in the laboratory. The laboratory mixing of the solid propellant was performed to approximate as closely as possible the actual production procedure. To each gram of propellant component were added 6×10^5 spores, contained on 10 mg of glass. The amount of each component required to make a propellant batch of 35 g was aseptically weighed into sterilized aluminum weighing pans and covered with another sterilized pan. All ingredients were warmed to 60 C in a hot-air oven for 2 hr to increase the pliability when mixing. The ammonium perchlorate was added to the PBAN polymer in three equal portions, with a total mixing time of 40 min. The EPON was then added, mixed, and placed at 71 C for 72 hr to cure. Samples were removed at each step of the procedure for bacteriological testing. The results of two individual experiments are presented in Table 4. Steps 1a through 1d test the recovery of viable spores after minimal exposure to the propellant compounds. These results are in agreement with those presented in Table 3. Exposure of the spore-propellant components (steps 2a–d) to 60 C for 2 hr appears to produce a reduction of 50 to 90% in the number of viable spores. During the mixing procedure represented in steps 3 to 6, the recovery of spores was reduced to the level of the PBAN of step 2d. During the total mixing period of 110 min, no evidence of a decrease in viability was observed. The curing

TABLE 4. *Spore recovery from a solid propellant at each formulation step**

Step	Component	Treatment	Temp	Time	Viable spores/g of propellant	
					Expt 1	Expt 2
			C			
1a	NH_4ClO_4, 48 mesh	None	Ambient	0	$>10^5$	3.4×10^5
1b	NH_4ClO_4, 10 μ	None	Ambient	0	$<10^2$	6×10^5
1c	EPON	None	Ambient	0	4.8×10^2	3×10^3
1d	PBAN	None	Ambient	0	3.7×10^3	2×10^3
2a	NH_4ClO_4, 48 mesh	Heat for consistency	60	2 hr	$>10^5$	2×10^5
2b	NH_4ClO_4, 10 μ	Heat for consistency	60	2 hr	$>10^5$	4.4×10^5
2c	EPON	Heat for consistency	60	2 hr	9×10^2	2×10^2
2d	PBAN	Heat for consistency	60	2 hr	$<10^2$	3.2×10^2
3	2d + ⅓(2a + 2b)	Mixing	Ambient	15 min	2.7×10^2	3.5×10^2
4	3 + ⅓(2a + 2b)	Mixing	Ambient	10 min	1.3×10^3	5×10^2
5	4 + ⅓(2a + 2b)	Mixing	Ambient	10 min	6.6×10^2	5.8×10^2
6	5 + 2c	Mixing	Ambient	15 min	$<10^2$	1.2×10^3
7	6	Curing	71	72 hr	4.8×10^2	2×10^2
8	7	Sterilization	135	24 hr	1	0

* Spores (6×10^5) on 10 mg of glass added per gram of each propellant component at step 1.

step, when the complete mixture is maintained at 71 C for 72 hr, caused no appreciable reduction in viability. Exposure of the cured propellant-spore mixture to 135 C for 24 hr reduced the number of recoverable viable spores practically to zero.

Field testing of solid propellant. A solid propellant was prepared on a pilot-plant scale and inoculated with 10^4 spores of *B. subtilis* per gram of final propellant mixture. This material was tested after the curing cycle for viable spores; no viable spores were recovered. This pilot-plant material had a more rubbery texture than did material prepared in the laboratory, and mixing of the material for testing was more unsatisfactory than the laboratory samples.

DISCUSSION

The ability to establish the sterility of rocket propellants is important in our design of interplanetary space vehicles. Ignition of the propellant mixture for propulsion generates sufficient heat to kill all living organisms. However, the isolated propellant components and unreacted mixtures are potential sources of interplanetary contamination. These systems operate in the complete absence of water, and valid exposure and test procedures should duplicate these conditions as closely as possible. The dry spore-glass powder procedure combined with vacuum evaporation was satisfactory for volatile compounds. With the use of these techniques, the entire exposure period was performed in the complete absence of water. Testing of nonvolatile components was less satisfactory. The number of viable spores was determined by rapid extraction with water, and then separation of the spores from the propellant compound by membrane filtration. For a brief period, the spores were exposed to the propellant compound in the presence of water. This exposure caused no noticeable killing. Occasionally, residual propellant on the filters gave evidence of toxicity during the growth period on nutrient agar. For this reason, results for the solid propellant material may give low values for the number of viable spores.

Interpretation of results from viscous or resinous materials requires great caution. Results from pilot-plant manufacture of the solid propellant would indicate that all spores were killed. However, examination of the individual components and the temperatures and reactions at each step of the formulation give no indication of killing effects. In reality, the solid propellants are a suspension of chemicals in a solid resin. All results of the experiments on the solid propellants are consistent with the interpretation that the viable spores have been encapsulated within the resin of the propellant. These spores are still viable and a possible source of contamination. EPON Resin 828 contains substituted ethylene oxide and bis-phenol structures. Ethylene oxide is an effective sporicide (Phillips and Kaye, 1949). Many of the bis-phenols, in particular those having halogen substitutions, are effective bacteriostatic and fungistatic compounds (*see* Reddish, 1957). However, the EPON Resin 828 shows no sporicidal properties under the test conditions.

Nitrogen dioxide was found to be an effective sporicidal compound. These results agree with those of Shank, Silliker, and Harper (1962), who found that the small amount of nitrogen dioxide present in nitric oxide was responsible for its bactericidal and sporicidal properties.

Zsolnai (1962) reported that UDMH is fungistatic at concentrations of 1:5,000. Our results show that monomethyl hydrazine and unsymmetrical dimethyl hydrazine are sporicidal in the absence of water when the exposure time is of the order of several days.

These test results show that the liquid rocket propellant systems tested are self-sterilizing in tests with *B. subtilis* and that the solid propellant systems tested require additional treatment to render them sterile.

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